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13. ABSTRACT (<i>Maximum 200 Words</i>) Germline mutations in the BRCA1 tumor suppressor gene have been implicated in hereditary breast and ovarian cancers. As part of our ultimate goal to characterize novel interactions between BRCA1 and other proteins involved in well-defined pathways, we have pursued an area of research that is just beginning to receive a great deal of attention for BRCA1 function, transcriptional regulation. Since BRCA1-associated cancers typically involve steroid hormone responsive tissues, it is possible that BRCA1 plays a role in steroid receptor signaling. Using transient transfection assays, BRCA1 was found to enhance androgen signaling via activation function-1 (AF-1) of the androgen receptor (AR). Furthermore, coexpression of BRCA1 with p160 nuclear receptor coactivators SRC1a, GRIP1, or AIB1 resulted in synergistic potentiation of androgen receptor and estrogen receptor (ER- α) signaling in both breast and prostate cells. The N-terminal subdomain of BRCA1 physically interacted with both the N-terminal domain of the AR and the C-terminal domain of the p160 nuclear receptor coactivator, GRIP1, by <i>in vitro</i> protein binding and mapping assays. Though further <i>in vivo</i> studies are necessary to determine the physiological significance of these interactions, these results suggest that BRCA1 may play a role in hormone regulation by directly modulating nuclear receptor-p160 coactivator interactions. Therefore, in fulfillment of our ultimate goals, we demonstrate a novel role for BRCA1 in transcriptional regulation which may aid in our understanding of how loss of BRCA1 results in increased neoplastic transformation.			
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INTRODUCTION

BRCA1 is a tumor suppressor gene found to be mutated in 30-45% of hereditary breast and ovarian cancers (1). It encodes a nuclear phosphoprotein with putative roles in DNA repair, cell-cycle control and transcriptional regulation (2-4). While mutations in *BRCA1* confer increased risk for both early-onset breast and ovarian cancers, their role in prostate cancer is controversial (5-7). Moreover, since the same germline *BRCA1* mutation exists in all tissues of an affected individual, it is not clear why breast and ovarian tissues are preferentially affected by neoplastic disease. The recent finding that *BRCA1* inhibits estrogen receptor (ER- α) signaling (8) suggests one mechanism by which loss of *BRCA1* function may lead to increased breast cancer risk in some carriers, but it does not explain all aspects of the disease seen in affected women.

The androgen receptor (AR) is a member of the nuclear receptor (NR) superfamily of transcriptional regulators, which includes the steroid, thyroid hormone and retinoic acid receptors. These receptors function in enhancing promoter-specific gene expression through the recruitment of multiple coregulatory proteins (ie. coactivators/corepressors) involved in various aspects of transcriptional control (9, 10). The AR shares a basic structural homology with the other NRs containing: an N-terminal transactivation domain (NTD), a highly-conserved DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (11). Both the NTD and the LBD contain separate activation functions (AF-1 and AF-2, respectively) that mediate the transcriptional action of the receptor. However, unlike other Class I steroid hormone receptors, AR also contains two, N-terminal poly-amino acid stretches encoded by polymorphic trinucleotide repeats. One of these is a highly-variable CAG-microsatellite which encodes polyglutamine (poly-Q). Although deleterious expansion of the microsatellite (≥ 40 CAGs) does occur, resulting in a rare, neurodegenerative disorder called spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, the normal CAG size-range in the general population is between 9 to 29 CAG repeats (12, 13) and averages about 20, depending on the population ethnic group (14). Variation in CAG-repeat length inversely modulates AR activity (13, 15), and shorter CAGs are correlated with both increased risk and earlier age-of-onset for prostate cancer (16), supporting the hypothesis that androgens play a direct role in promoting prostate cancer development (17). Yet, despite this role in the prostate, androgens may play a protective role in breast, possibly through AR-mediated inhibition of breast epithelial cell proliferation (18, 19). This is supported by the fact that some mutations in the AR leading to loss of function or expression have been correlated with breast cancer and earlier age-of-onset in some individuals, though the frequency of such alterations are rare and not well-characterized (20-23). Recently, a correlation between CAG-repeat length and risk for breast cancer development was observed in a cohort of women with known *BRCA1* mutations (24). In that study, women who were *BRCA1* mutation carriers and had at least one AR allele with ≥ 28 CAG repeats were more likely to develop breast cancer earlier than similar age-matched controls. In a separate study of women who developed sporadic breast cancer by age of 40 years, no significant correlation with CAG repeat size and cancer risk was found (25). Therefore, since variation in AR activity, as determined by CAG repeat size, affected *BRCA1*-associated breast cancer risk, but not sporadic breast cancer risk, it is possible that *BRCA1* may function either directly, or indirectly, in regulating AR signaling. Together, these observations suggest a likely functional connection between the AR and *BRCA1*.

MATERIALS AND METHODS

Transient transfection assays were performed using a chloramphenicol-acetyl transferase (CAT) assay system. Mammalian cells were transfected with plasmids encoding various transcription factors and tested for reporter gene activation. In some cases, protein expression from transfected plasmids were checked by immunofluorescence. Protein binding assays were performed using glutathione-S-transferase (GST) fusion proteins and *in vitro* transcribed and translated protein targets.

Mammalian Expression Vectors and Plasmid Construction

Plasmids pCMV-hAR (26), pSG5-ER α (27), pSG5-GRIP1 and pSG5-SRC-1a (28), pcDNA3.1-AIB1 (29), ARR $_3$ tk-CAT (30), ERE-Coll60-CAT (31), and MMTV-CAT (32) were described previously. To construct the vector pcDNA-AR (NTD-DBD), an NheI-BamHI fragment was PCR amplified from pcDNA-hAR (33) plasmid DNA using AR (NTD-DBD) primer pairs S1 and AS1 (Table 1) and inserted into the reciprocal restriction sites of pcDNA3.1 (+). Vector pcDNA-AR (DBD-LBD) was constructed in sequential cloning steps. First, an NheI-KpnI PCR fragment containing the AR Kozak sequence was amplified using primers S1 and AS2 and inserted into the corresponding sites of pcDNA3.1 (+). Second, a KpnI-EcoRI PCR fragment was amplified using primers S2 and AS3 (Table 1) and inserted into the restored KpnI site and the downstream EcoRI site of the pcDNA3.1 (+) multiple cloning site. BRCA1 mammalian expression plasmid pcDNA-BRCA1 was constructed by inserting a 5' NotI-XhoI 3'-treated BRCA1 insert derived from pBSK-1hFL plasmid (34) into corresponding endonuclease restriction sites of a pcDNA3.1/mycHisC(-) vector (Invitrogen).

Overlapping KpnI-XhoI BRCA1 fragments were amplified by PCR using the indicated primer pairs (Table 1). Forward primers contain engineered KpnI restriction endonuclease sites followed by a SV40 T antigen nuclear localization signal (NLS). Reverse primers contain a hemagglutinin A (HA) tag followed by a novel XhoI site. Elongase PCR (Life Technologies, Rockville, MD) amplified BRCA1 fragments were purified by gel extraction (Qiagen, Valencia, CA), double-digested with KpnI-XhoI and inserted into a pcDNA3.1 (Kozak) vector.

Bacterial Expression Plasmids

Bacterial expression plasmids encoding GST, GST-AR and GST-GRIP1 fragments were previously described (4, 28).

Tissue culture and transfections

Cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in RPMI (PC-3, DU-145, and HBL-100) or DMEM (MCF-7) medium that contained 10% fetal bovine serum (FBS). Approximately 24 h prior to transfection, 10⁶ (PC-3, DU-145, and HBL-100) or 5 x 10⁵ (MCF-7) cells were seeded into each 60-mm dish. Cells were transfected in serum-free conditions with Lipofectamine reagent (Life Technologies, Rockville, MD) according to the manufacturer's protocol. In each experiment, the total amount of DNA per dish was held constant by the addition of pcDNA3.1 (+) vector when appropriate (Invitrogen, Carlsbad, CA). Following transfection, cells were grown for 24 h (DU-145, HBL-100, and MCF-7) or 48 h (PC-3) in RPMI medium (without phenol red) that contained 5% charcoal/dextran-stripped FBS (Gemini Bio Products, Calabasas, CA) and, where indicated, DHT (1 or 10 nM) or 10 nM E2 for the last 24 h of growth. Whole-cell extracts were prepared in 0.25 M Tris-HCl pH 8.0 by repeated freezing and thawing. CAT assays were performed using the Quan-T-CAT kit (Amersham Pharmacia Biotech, Piscataway, NJ) (see below) and total cellular protein was measured using the BioRad (Hercules, CA) Protein Asssay kit. Relative CAT activities (c.p.m./O.D.₆₀₀) are reported as the mean ± SE of three independent dishes.

Chloramphenicol Acetyltransferase (CAT) Assays

Cell extracts were prepared as described above. 40 μ l amounts of cell extracts were transferred into 1.5 ml microcentrifuge tubes. 10 μ l of substrate mix was added to each tube and incubated at 37°C for 30 minutes. After incubation, 1 ml of diluted bead mix was added to each reaction tube and allowed to stand at room temperature for 5 minutes. Beads were then spun down into tight pellets by centrifugation for 5 minutes and radioactive supernatant was collected and discarded. Pellet was washed with 1 ml of wash buffer and then respun for 3-5 minutes. Supernatants were again discarded and 1 ml of scintillation cocktail was added to each tube. Pellets were mixed by brief vortexing and reactions were counted and analyzed..

Glutathione-S-Transferase (GST) Pull-Downs

Glutathione-S-transferase (GST) and GST-fusion proteins were expressed and purified as described (35). Glutathione-Sepharose-bound GST protein, GST-AR (1-555), or GST-GRIP1 fragments (5-765, 563-1121, or 1121-1462) were incubated with 35 S-radiolabeled full-length BRCA1 or BRCA1 fragments transcribed and translated *in vitro* from pcDNA3.1 vectors using a TNT-Coupled Reticulocyte Lysate System (Promega) in the presence of 35 -S methionine. Associated BRCA1 was eluted, resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by autoradiography. 10% of total labeled BRCA1 incubated in each reaction was loaded for comparison.

RESULTS

BRCA1 enhances AR signaling

In order to assess the role of BRCA1 in AR signaling, we cotransfected PC-3 prostate cancer cells with a wild-type *AR* expression vector and increasing amounts of a wild-type *BRCA1* expression vector. Androgen-dependent activation of AR was enhanced by coexpression of exogenous BRCA1 (Figure 1). Substitution of the *BRCA1* plasmid with parent vector (pcDNA3.1) failed to generate a coactivation response indicating that functional BRCA1 is required. No coactivation by BRCA1 was observed in the absence of DHT (Figure 2-1). Furthermore, BRCA1 failed to stimulate the reporter gene in the absence of exogenous AR (data not shown).

Exogenous BRCA1 does not increase AR stability

The androgen receptor is highly stabilized by DHT (approximately 2-5 fold). To test if BRCA1 enhancement of AR signaling is biochemical and not due to stabilization, we transfected CV-1 cells with AR alone (no DHT versus 10 nM DHT) and with AR + BRCA1 (10 nM DHT). As a control baseline, we used untransfected CV-1 cells. Following transfection, the cells were incubated for 48 hours, lysed in RIPA buffer, and the extracted lysates were then quantified by BCA protein quantification assay. 7.5 µg of total lysates were resolved on a 4-20 % gradient gel and transferred onto a PVDF membrane. Membranes were blotted with anti-AR antibodies and detected by ECL chemiluminescent reagent. Untransfected cell lysates contained no detectable AR, whereas transfected cell lysates all demonstrated AR bands. As expected, DHT stabilized AR approximately 4 fold. However, coexpression of BRCA1 in the presence of DHT did not result in a statistically significant increased AR stability over DHT alone (Figure 2). Hence, the 2-3 fold observed enhancement of AR activity by BRCA1 cannot be attributed to a stabilization phenomenon.

BRCA1 enhances AF-1 of the AR

Full-length BRCA1 was coexpressed in PC-3 cells with either a constitutively active AR variant comprising the NTD and the DBD, i.e. AR(NTD-DBD), to assess AR AF-1 activity, or a variant comprising the DBD and LBD, i.e. AR(DBD-LBD), to assess AR AF-2 activity. BRCA1 enhanced the activity of AR(NTD-DBD) but was unable to activate AR(DBD-LBD) in either the presence or absence of ligand (Figure 3). Therefore, BRCA1 is able to coactivate the AF-1 function of the AR.

Potentiation of p160 coactivation by BRCA1

The p160 coactivators (i.e. SRC1/NcoA1, GRIP1/TIF2/NcoA2, and AIB1/p/CIP/ACTR) are nuclear proteins that bind to NRs and potentiate ligand-dependent receptor signaling by recruiting transcriptional regulatory proteins, including histone acetyltransferases (9, 10) and methyltransferases (4). They interact with, and coactivate the AR through both AF-1 and AF-2 of the receptor (28). To determine if BRCA1 is involved in p160-mediated coactivation of AR signaling, we cotransfected mammalian cell lines with expression vectors for BRCA1 and/or GRIP1, SRC-1a, or AIB1 along with AR. BRCA1 and the p160 coactivators individually were able to enhance AR activity in PC-3 cells in a hormone-dependent fashion (Figure 4). Coexpression of BRCA1 with each of the p160 coactivators resulted in synergistic coactivation of AR signaling (Figure 4), results that were duplicated using GRIP1 and BRCA1 in a second prostate cancer cell line (DU-145), and two breast cell lines (HBL-100 and MCF-7) (Figure 5). The relatively small effects observed in MCF-7 cells may be due to the overexpression of endogenous AIB1 (29).

BRCA1 enhances GRIP1-mediated coactivation of AR LBD

Since BRCA1 was able to potentiate p160-mediated coactivation of wild-type AR, we argued that it should be able to do the same with the AR LBD. As expected, in transiently transfected PC-3 cells, hormone-dependent reporter gene activation by the AR(DBD-LBD) was enhanced by GRIP1 coexpression (Figure 6). Furthermore, this activity was potentiated by coexpression of BRCA1, although BRCA1 had little or no effect on AR(DBD-LBD) signaling in the absence of GRIP1 coexpression. Similar results were observed when AIB1 was substituted for GRIP1 (data not shown). Therefore, BRCA1 can coactivate AR AF-2, but only in the presence of co-associated p160 coactivators.

BRCA1 interacts with the N-terminus of AR and the C-terminus of GRIP1

The ability of BRCA1 to coactivate AR and to potentiate p160-mediated enhancement of AR signaling suggests physical interactions. To determine if BRCA1 physically associates with the AR and/or GRIP1, *in vitro* protein binding experiments were performed using glutathione-S-transferase (GST)-fused fragments of either AR or GRIP1 and radiolabeled full-length BRCA1. Schematic representations of the AR, BRCA1 and GRIP1, including major domains and motifs, are shown (Figure 7). In these experiments, full-length BRCA1 was found to interact specifically with both GST-AR (1-555) (Figure 8) and GST-GRIP1c (1121-1462) (Figure 9). No interactions were detected with GST-GRIP1 (5-765, 563-1121) or GST-protein alone.

Both AR and GRIP1 interact with the N-terminus of BRCA1

In order to map the interactions of both the AR-NTD and GRIP1c onto BRCA1, ³⁵S-radiolabeled, overlapping fragments of BRCA1 were incubated with GST-AR (1-555) or GST-GRIP1c (1121-1462) and analyzed as described above. Pull-down results localize both AR and GRIP1 interactions to a region in the N-terminus of BRCA1 spanning amino acids 1-404 (Figure 10). This region of BRCA1 contains a cysteine-rich zinc-binding domain, or RING finger, which is believed to function in protein-protein interactions (1). However, it is not determined if this RING finger is important in mediating AR and/or GRIP1 binding.

BRCA1 coimmunoprecipitates with GRIP1, but not with GRIP1 Δ AD2

To test if BRCA1 and GRIP1 interact in mammalian cells, we cotransfected SV40-transformed COS-7 monkey kidney cells with BRCA1 and either HA-GRIP1 or HA-GRIP1 Δ AD2, which lacks the AD2 interaction domain. As a negative control, untransfected cells were grown simultaneously. Whole cell lysates (WCL) were immunoprecipitated with either a BRCA1 polyclonal antibody (C-20, Santa Cruz), or a non-specific rabbit polyclonal antibody (rabbit IgG, Zymed). Immune complexes were stringently washed and eluted proteins were resolved by SDS-PAGE and transferred onto a PVDF membrane. Membranes were probed for coimmunoprecipitated HA-GRIP or HA-GRIP Δ AD2 using a rat anti-HA antibody (Roche Pharmaceuticals). Full-length HA-GRIP1, but not the truncated Δ AD2 mutant, coimmunoprecipitated with BRCA1 (Figure 11). This interaction was specific for BRCA1 as no coimmunoprecipitated HA-GRIP1 was detected when normal rabbit IgG was used. Likewise, no detectable band was seen in the untransfected negative control. To demonstrate that these results were not due to differences in HA-GRIP1 or HA-GRIP1 Δ AD2 expression, samples of each WCL used for the coimmunoprecipitation assays were screened by western blotting to detect HA-tagged proteins in transfected versus untransfected cells (Figure 11). Using a rat anti-HA antibody, appropriate bands of expected molecular weights were detected in each of the samples derived from transfected cells, but no bands were observed in the untransfected cells.

BRCA1 synergy with GRIP1 is dependent upon an intact AD2 domain

Since BRCA1 was unable to interact with the GRIP1 Δ AD2 mutant by GST pull-down assay and coimmunoprecipitation, we wanted to test if this loss of interaction resulted in a corresponding loss of functional synergy. In CV-1 cells, we transfected AR, BRCA1 and combinations of either HA-GRIP1, HA-GRIP1 Δ AD1, or HA-GRIP1 Δ AD2. Luciferase activities were assayed using the methods described above [Methods]. Both HA-GRIP1 and HA-GRIP Δ AD1 were able to enhance BRCA1 activity on AR signaling. HA-GRIP1 Δ AD2, however, was unable to function with BRCA1, suggesting that BRCA1 and GRIP1 synergy is dependent upon direct interaction between the two coactivators (Figure 12).

Key Research Accomplishments:

- Cloned mammalian expression plasmids of BRCA1, the p160 nuclear receptor coactivators (GRIP1, SRC1a, AIB1), the nuclear receptors (AR, ER, PR), and the protein methyltransferases (CARM1, PRMT1) for *in vitro* interaction assays and functional studies.
- Characterized novel protein-protein interactions illustrating a role for BRCA1 in various aspects of transcriptional regulation: (1) BRCA1 and the androgen receptor and (2) BRCA1 and the p160 family of nuclear receptor coactivators.
- Mapped the interaction domains for BRCA1-AR and BRCA1-GRIP1 to the N-terminus of BRCA1 for both AR and GRIP1, and to the N-terminus of AR and the C-terminus of GRIP1 for BRCA1.
- Demonstrated physical association between BRCA1 and GRIP1 in mammalian cells by coimmunoprecipitation assays.
- Characterized BRCA1 function as a coactivator of the steroid receptors (AR and ER), both alone and in the presence of the p160 coactivators. Coactivation synergy between BRCA1 and GRIP1 was described in multiple, mammalian cell lines for both AR and ER signaling pathways.

Reportable Outcomes:

Manuscripts:

1. **John J. Park**, Ryan A. Irvine, Grant Buchanan, Stephen S. Koh, Jinha M. Park, Wayne D. Tilley, Michael R. Stallcup, Michael F. Press, and Gerhard A. Coetzee. BRCA1 is a Coactivator of the Androgen Receptor. *Cancer Res.* (Submitted, 2000).

Poster Presentations:

1. **John J. Park**, Ryan A. Irvine, Stephen S. Koh, Jinha M. Park, Gerhard A. Coetzee, and Michael F. Press. BRCA1 coactivators both AR and ER signaling in breast and prostate cancer cell lines. Era of Hope, Department of Defense Breast Cancer Research Program, Atlanta, GA, on June 9, 2000. Abstract in the Department of Defense Breast Cancer Research Program *Proceeding*, v.I: 69 (2000).

Presentations:

1. Invited Speaker, The Bob Rope Foundation Breast Cancer Research Program, Palm Springs, CA, March 30-31, 2000. Title: BRCA1, Molecular Interactions and Functions.

Patents and Licenses:

(Not applicable)

Doctoral Degree In Progress:

John J. Park will be completing the requirements for the Doctor of Philosophy degree on September 12, 2000, which upon successful defense of his thesis, will be conferred simultaneously with his MD in May 2002 at the Keck School of Medicine, University of Southern California.

Development of Cell Lines and Novel Recombinant Genes:

A stable BRCA1-overexpressing HBL-100 breast epithelial cell line was generated using G418 selection. The cell line has not been fully characterized and has not be reported in a public forum.

Personnel Receiving Pay for this Research Effort:

John J. Park, Pre-doctoral Trainee

CONCLUSIONS

Several independent lines of evidence suggest that BRCA1 may play a role in transcriptional regulation. BRCA1 contains a transcriptional activation domain (36, 37), that functions as a coactivator of p53-dependent gene expression (38-40) and has been shown to be associated with the RNA polymerase II holoenzyme complex (41, 42). The recent finding that BRCA1 inhibits ER activity (8) suggested, at that time, that BRCA1 may protect against breast cancer risk due to this inhibition. In conclusion of this study, we now demonstrate that BRCA1 enhances androgen-responsive reporter gene expression by interacting with, and activating AF-1 of the AR NTD. Furthermore, BRCA1 can potentiate the effect of p160 coactivators on AR signaling in breast and prostate cell lines, possibly by modulating nuclear receptor-p160 coactivator interactions. Interestingly, both the AR and the p160 coactivators appear to interact with the N-terminus of BRCA1. This is also the region of BRCA1 where both p53 (38-40) and *c-myc* binding occurs (43), revealing a novel feature of this relatively small, but important, region. It is possible that BRCA1 potentially modulates transcription by stabilizing interactions between transcription factors associated with its N-terminus and the transcriptional initiation complex bound to its C-terminus. Indeed, deletion of this C-terminal interaction domain results in the loss of BRCA1's coactivation function as it can no longer recruit the preinitiation complex to sites of active gene transcription (38-40). Therefore, mutations which lead to premature truncation of BRCA1 likely result in defective, or impaired, function due to loss of this bridging interaction, though presumably, BRCA1's ability to associate with transcriptional regulatory proteins through its N-terminus is unaffected. Thus, it is possible that truncated forms of BRCA1 may act as dominant negative inhibitors in heterozygous mutation carriers resulting in significant dysregulation of transcription by sequestering limiting factors, thereby, diminishing the effectiveness of the remaining wild-type BRCA1 protein.

The results of this study suggest a complex interplay between AR, the p160 coactivators and BRCA1 in modulating cell proliferation, and by implication cancer risk, in tissues like the prostate and breast. In prostate, loss of BRCA1 was initially thought to be associated with an increased risk for developing cancer, although later studies considering specific mutations failed to demonstrate any such correlation in mutation carriers (5-7). In retrospect, this is not surprising since BRCA1 appears to function as a coactivator of AR signaling. As such, the loss of BRCA1 might, in fact, protect against prostate cancer by decreasing AR activity.

In the case of breast cancer risk, we have uncovered novel interactions that may ultimately aid in our understanding of how BRCA1 associated cancer may arise. In women with only one functional copy of BRCA1 exhibit a significant correlation between AR-CAG size alleles and early-onset disease (24), suggesting that BRCA1 acts to maintain AR's protective effect on the breast by potentiating AR activity during normal signaling events. According to this model, when there is partial loss of BRCA1 function, AR signaling might be significantly diminished, potentially leading to greater breast cell proliferation. The fact that both lower AR activity and *AIB1* alterations in BRCA1 mutation carriers result in similar cancer phenotypes and risk profiles supports this hypothesis. Although further studies are required, these findings may help in our understanding of the role of BRCA1 in breast cancer development, and may be useful in remodeling current treatment protocols based on the unique biology of BRCA1-associated cancers. In continuation of the research activities developed over the course of this scientific endeavor, our laboratory, in collaboration with other laboratories, are pursuing other avenues of BRCA1 function in transcriptional regulation, such as chromatin remodeling.

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APPENDICES

A. Figures

Figure 1. Wild-type BRCA1 coactivates AR transactivation in PC-3 prostate cancer cells.

Transiently transfected cells were assayed for stimulation of ARR₃-tk-CAT reporter activity by dihydrotestosterone (DHT). ARR₃-tk-CAT is composed of a minimal thymidine kinase (TK) promoter under the control of three identical fragments of the rat probasin promoter (nucleotides -244 to -96) each comprising two androgen responsive elements (i.e., ARBS-1 and ARBS-2) (30). Cells were cotransfected with 2.0 µg ARR₃-tk-CAT, 50 ng pCMV-hAR, and increasing amounts of pcDNA-BRCA1 as indicated. Total transfected DNA was held constant by the addition of pcDNA3.1 vector when appropriate. Chloramphenicol acetyl transferase (CAT) activities were normalized for total cellular protein and data presented are the mean + SE of three independent dishes. Fold is measured relative to DHT-dependent AR activity with no transfected BRCA1.

Figure 2. AR is not stabilized by BRCA1 coexpression. 5x10⁵/well PC-3 prostate cancer cells were grown overnight on 24-well tissue culture plates. Next day, cells were transfected with the following amounts of plasmids, as described above: 50 ng CMV-AR, 250 ng pcDNA3.1-BRCA1. Transfections were performed in triplicates using Superfect reagent (Gibco-BRL) as per Superfect protocol. Cells were then incubated for 48 hours either in the absence or presence of 10 nM DHT. After the incubation time, lysates were harvested in RIPA buffer and quantified by BCA Protein Quantification Assay (Pierce). 7.5 µg of total lysates/sample were loaded onto a 4-20% gradient gel (Bio-Rad), resolved by SDS-PAGE, and then transferred onto a PVDF membrane. Western immunoblot analysis was performed using a rabbit-anti(AR) polyclonal antibody (1mg/ml) (Santa Cruz). Gel Quantification and analysis was done using a GS-710 calibrated imaging densitometer (Bio-Rad). Bar graphs are shown as relative transmission O.D. (relative to AR, 10 nM DHT). There is no significant increase in AR band density observed in the presence of BRCA1.

Figure 3. BRCA1 works through AR AF-1 in PC-3 cells. Cells were cotransfected with 50 ng pCMV-hAR, 10 ng pcDNA-AR (NTD-DBD), or 0.5 µg pcDNA-AR (DBD-LBD), 2.0 µg ARR₃-tk-CAT, and 2.5 µg pcDNA-BRCA1 as indicated. Mammalian expression vectors pcDNA-AR (NTD-DBD) and pcDNA-AR (DBD-LBD) encode AR amino acids 1-647 and 538-919, respectively. AR (NTD-DBD) is a constitutive activator of ARR₃-tk-CAT and thus, potentiation of its activity by BRCA1 is ligand independent.

Figure 4. Synergistic coactivation of AR signaling by BRCA1 and members of the p160 family of nuclear receptor coactivators. PC-3 cells were cotransfected with 2.0 µg pSG5-GRIP1, pcDNA3.1-AIB1, or pSG5-SRC-1a, 2.0 µg ARR₃-tk-CAT, 25 ng pCMV-hAR, and 2.5 µg pcDNA-BRCA1 as indicated. In each case, AR transactivation activity in the presence of transfected BRCA1 and p160 coactivator was greater than the additive effects of BRCA1 and p160 coactivator assayed separately.

Figure 5. Potentiation of AR signaling by BRCA1 occurs in both prostate- and breast-derived cell lines. Prostate cell line DU-145 and breast cell lines HBL-100 and MCF-7 were cotransfected with 2.0 µg ARR₃-tk-CAT, 25 ng pCMV-hAR, 2.0 µg pSG5-GRIP1, and 2.5 µg pcDNA-BRCA1 as indicated. The data presented are fold + SE relative to DHT-dependent AR activity with no transfected BRCA1 or GRIP1. As in PC-3, a synergistic coactivation of AR signaling by BRCA1 and GRIP1 occurred in these cell lines. The relatively small effects observed in MCF-7 may be due to the overexpression of endogenous AIB1 in this cell line (29).

Figure 6. BRCA1 potentiates GRIP1-mediated coactivation of AR AF-2 on the MMTV promoter. PC-3 cells were transfected with 2.0 µg MMTV-CAT, 1.0 µg pcDNA-AR (DBD-LBD), 2.0 µg pSG5-GRIP1, and 2.5 µg pcDNA-BRCA1 as indicated. BRCA1 failed to coactivate AR AF-2 in the absence of exogenously expressed GRIP1 suggesting that it cannot make a functional contact with AR LBD. Since BRCA1 potentiates GRIP1-mediated coactivation of AR AF-2, it is likely that GRIP1 recruits BRCA1 through direct contacts.

Figure 7. Schematic diagrams of AR, GRIP1, and BRCA1 showing the locations of various functional domains. Domains of AR: AF-1/AF-2, autonomous activation functions 1 and 2; NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain; Q/P/G, glutamine/proline/glycine poly-amino acid stretches. Domains of GRIP1: bHLH, basic helix-loop-helix sequence; PAS, Per-Arnt-Sim domain; NR boxes, nuclear receptor binding domains (LXXLL motifs); CID, CBP interaction domain; AD1/AD2, activation domains. Domains of BRCA1: RING, zinc-finger domain; NLS, nuclear localization signals; BRCT, BRCA1 carboxy terminus. Numbers represent relative amino acid positions.

Figure 8. Full-length BRCA1 interacts with the N-terminal domain of the androgen receptor. Glutathione-S-transferase (GST) and GST-fusion proteins were expressed and purified as described (34). Glutathione-Sepharose-bound GST protein and GST-AR (1-555) were incubated with ³⁵S-radiolabeled BRCA1 transcribed and translated *in vitro* from pcDNA3.1 vector encoding full-length BRCA1. Associated BRCA1 was eluted, resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by autoradiography. 10% of total labeled BRCA1 incubated in each reaction was loaded for comparison.

Figure 9. Full-length BRCA1 interacts with the C-terminus of GRIP1. Glutathione-S-transferase (GST) and GST-fusion proteins were expressed and purified as described (34). Glutathione-Sepharose-bound GST protein or GST-GRIP1 fragments (5-765, 563-1121, or 1121-1462) were incubated with ³⁵S-radiolabeled BRCA1 transcribed and translated *in vitro* from pcDNA3.1 vector encoding full-length BRCA1. Associated BRCA1 was eluted, resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by autoradiography. 10% of total labeled BRCA1 incubated in each reaction was loaded for comparison

Figure 10. Autoradiographs showing the localization of the AR NTD and GRIP1c interactions on BRCA1. Plasmids containing fragments of BRCA1 were generated as described. Diagrams of the functional domains corresponding to amino acids are provided for reference. Unpurified *in vitro* translated BRCA1 fragments were incubated with GST, GST-AR (1-555) or GST-GRIP1 (1121-1462). Both AR and GRIP1 binding is shown to localize to the N-terminus of BRCA1.

Figure 11. BRCA1 coimmunoprecipitates with GRIP1, but not with GRIP1 Δ AD2. SV40-transformed COS-7 monkey kidney cells were transfected with HA-GRIP1 or HA-GRIP1 Δ AD2. Untransfected cell were grown simultaneously as a control. All samples were grown and treated in triplicates. Whole cell lysates were extracted, immunoprecipitated with either 1 mg/mL of specific rabbit anti-BRCA1 polyclonal antibodies (C20, Santa Cruz) or 1 mg/mL of non-specific rabbit polyclonal antibodies (Zymed), and washed with RIPA buffer (plus complete protease inhibitors, Roche Pharmaceuticals). Immune complexes were eluted in sample buffer and resolved by SDS-PAGE. Proteins were transferred onto PVDF membranes and probed for HA-GRIP1 or the Δ AD2 mutant using a rat anti-HA polyclonal antibody (Roche Pharmaceuticals).

Figure 12. Synergy with GRIP1 is dependent upon an intact AD2 domain. CV-1 cells were transfected with AR, BRCA1 and/or HA-GRIP1 plasmids. HA-GRIP1 Δ AD1 and HA-GRIP1 Δ AD2 mutants were substituted for HA-GRIP1 where indicated. GRIP1 and GRIP1

mutant effects on BRCA1 synergy were measured by luciferase assay and plotted with respect to AR and AR+BRCA1 activities.

B. Tables

(Refer to following page, Table 1)

TABLE 1

AR (NTD-DBD)	Primer Sequences Sense (S1) (5'-GTGGCAGCTAGCTGCAAGCGACTAC-3') Antisense (AS1) (5'-ATGGAGGGATCCCTCAGTGCTGGAAGGCCCTCCTTC-3')
AR (DBD-LBD)	Primer Sequences Sense (S2) (5'-AACTCGCGGTACCCGTTGGAGACTGCCAGG-GACCAT-3') Antisense (AS2) (5'-ACCTAAGGTACCCCCCTAAC TGCACTCCATCCT-3') Antisense (AS3) (5'-GGAAATGAATTGGGGAAATAGGGTTCCAAT-3')
BRCA1, 1-404	Primer Sequences Sense (5'-GCTAGGGTACCCCCAAGAAAGAAGCGTAAGATGGATTATCTGCTCTCGCGTT-3') Antisense (5'-GTCAGCTCGAGTCAAGCGTAAGCGTAATCTGGAACATCGTATGGTATTACCAATGAAATACTGCTACTCTC-3')
BRCA1, 364-772	Primer Sequences Sense (5'-GCTAGGGTACCCCCAAGAAAGAAGCGTAAGAGAGATACTGAAGATGTTCCCTGG-3') Antisense (5'-GTCAGCTCGAGTCAAGCGTAAGCGTAATCTGGAACATCGTATGGTATGCACTGACTACTCCCATAAGGCT-3')
BRCA1, 768-1142	Primer Sequences Sense (5'-GCTAGGGTACCCCCAAGAAAGAAGCGTAAGAGTATTTCATTGGCTGGTACTGAT-3') Antisense (5'-GTCAGCTCGAGTCAAGCGTAATCTGGAACATCGTATGGTATGCACTGACTACTCCCATAAGGCT-3')
BRCA1, 1085-1529	Primer Sequences Sense (5'-GCTAGGGTACCCCCAAGAAAGAAGCGTAAGTTAGGGGTTTGCAACCTGAGGTC-3') Antisense (5'-GTCAGCTCGAGTCAAGCGTAATCTGGAACATCGTATGGTAAATGAGCTCCTTGAGATGGGT-3')
BRCA1, 1490-1863	Primer Sequences Sense (5'-GCTAGGGTACCCCCAAGAAAGCGTAAGGAACCCAGGAGTGGAAAGGTCAATCC-3') Antisense (5'-GTCAGCTCGAGTCAAGCGTAATCTGGAACATCGTATGGTAGTGGCTGGGGATCTGGGT-3')

FIGURE 1

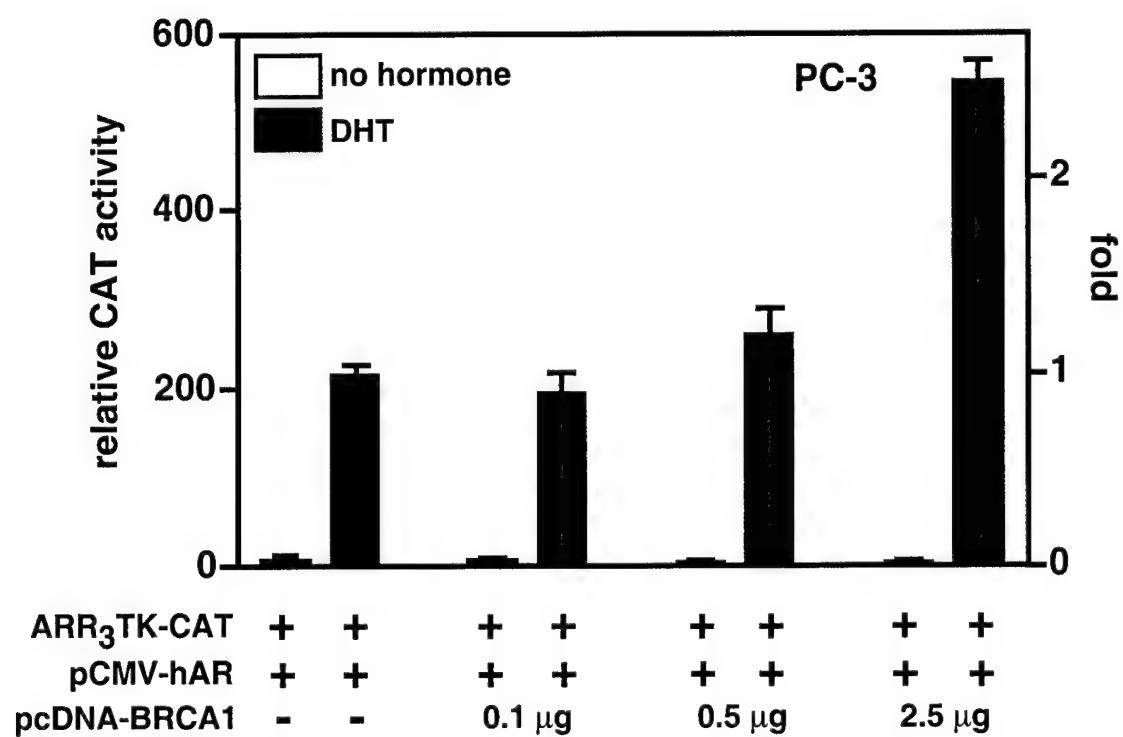


FIGURE 2

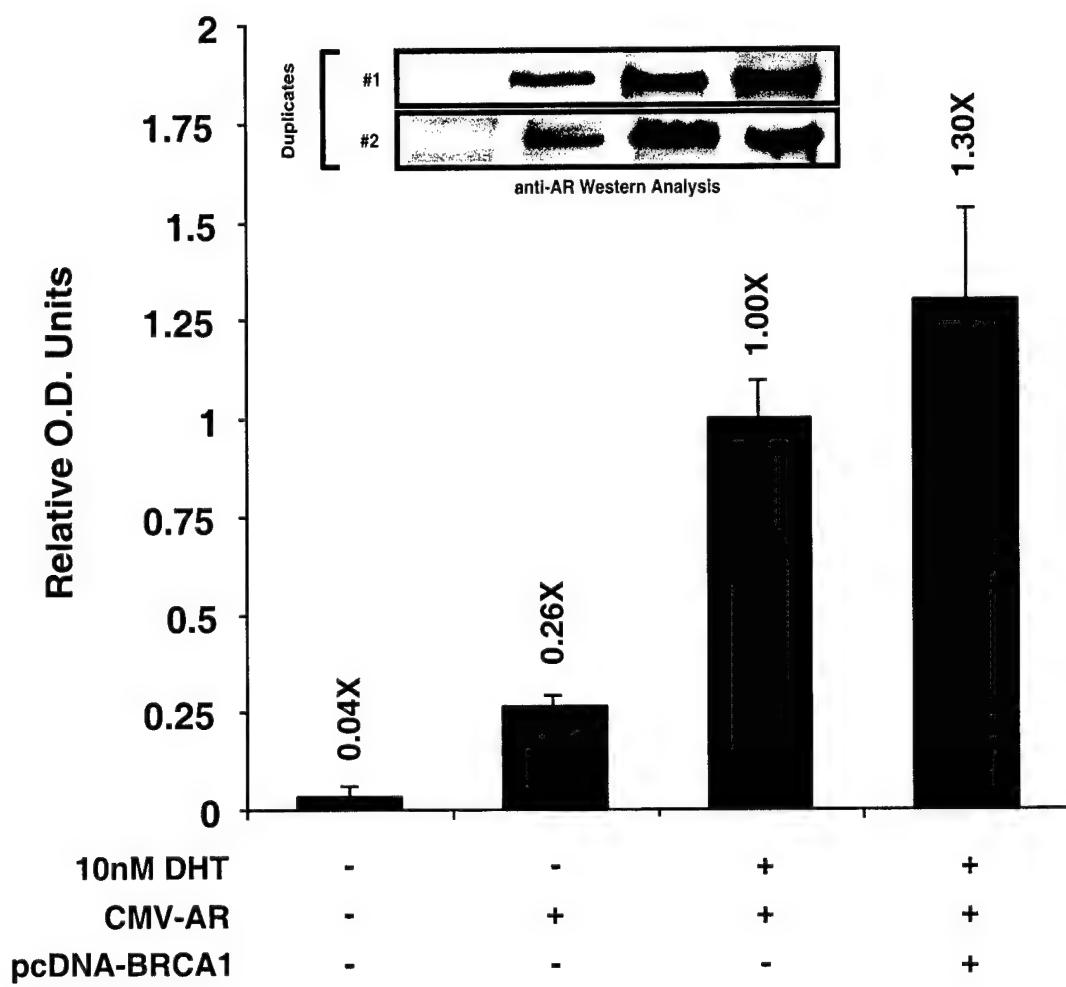


FIGURE 3

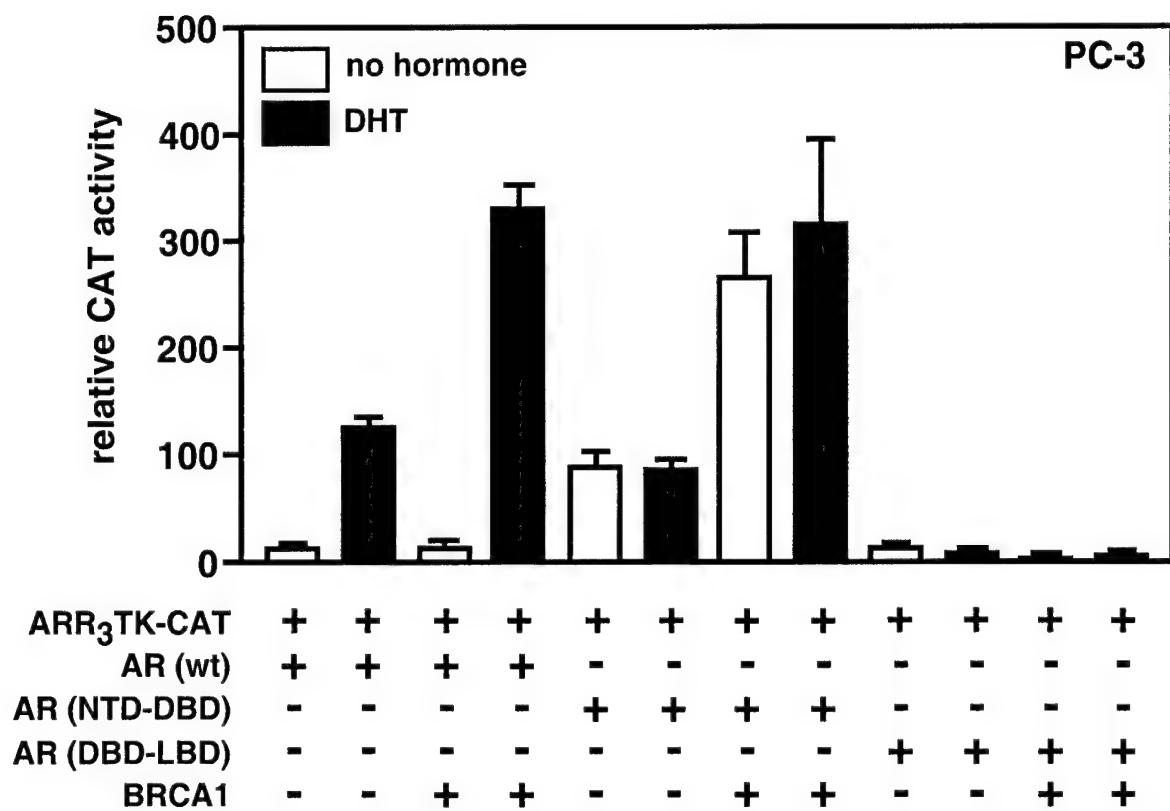


FIGURE 4

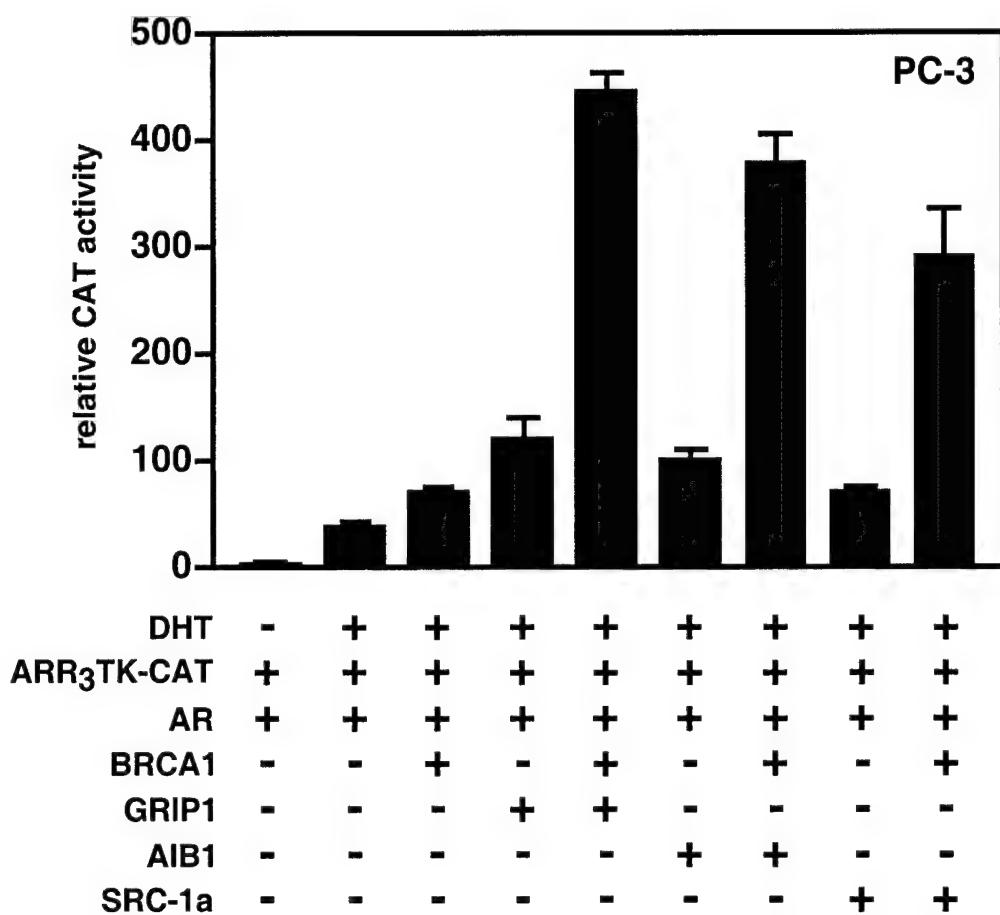


FIGURE 5

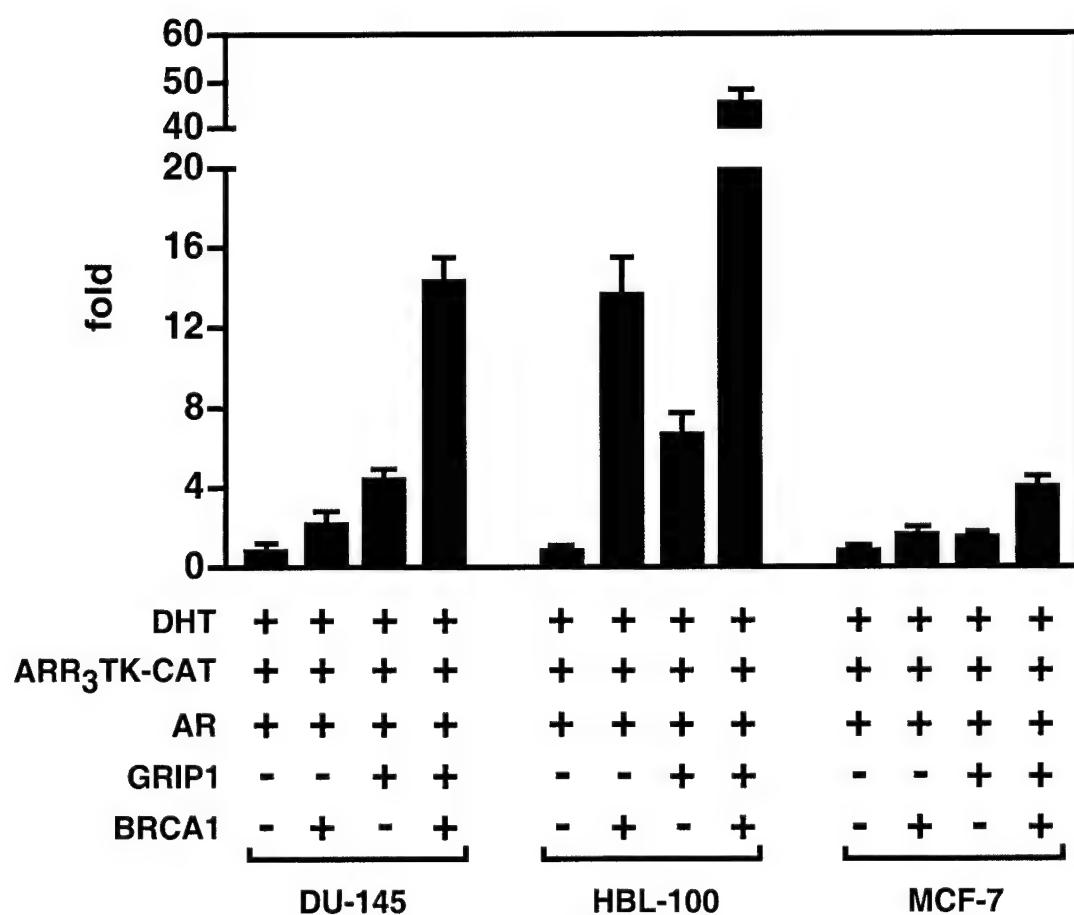


FIGURE 6

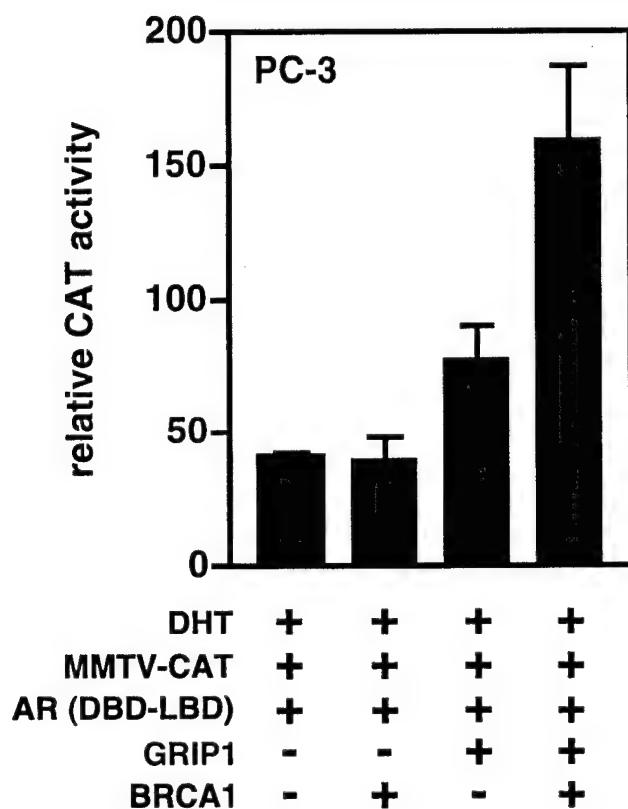
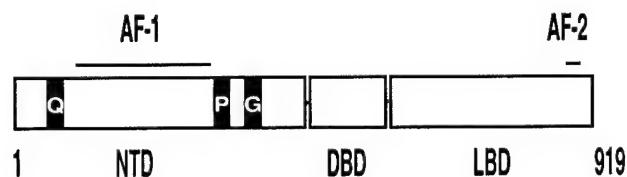
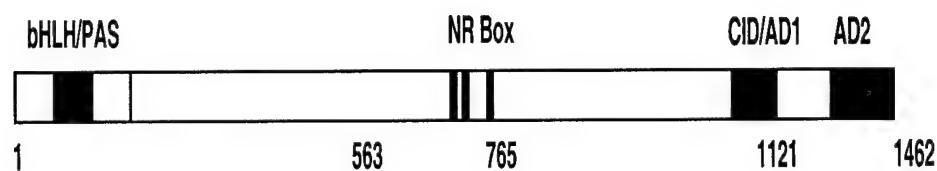


FIGURE 7

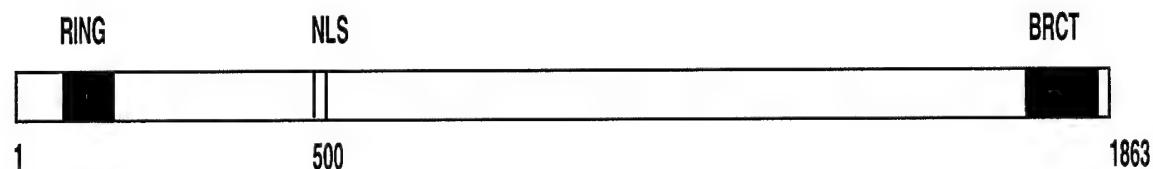
A. Androgen Receptor



B. GRIP1



C. BRCA1



FIGURES 8 & 9

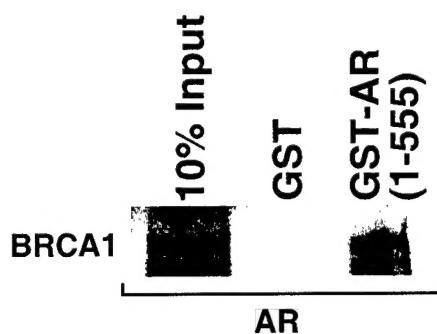
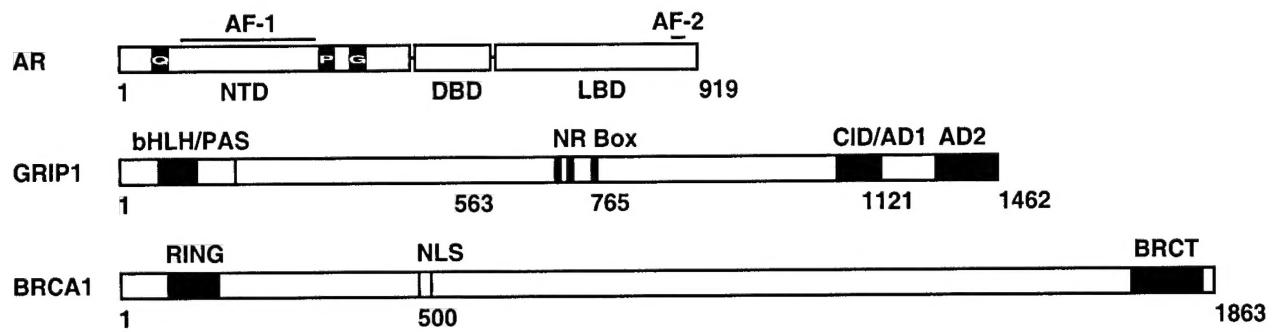


FIGURE 8

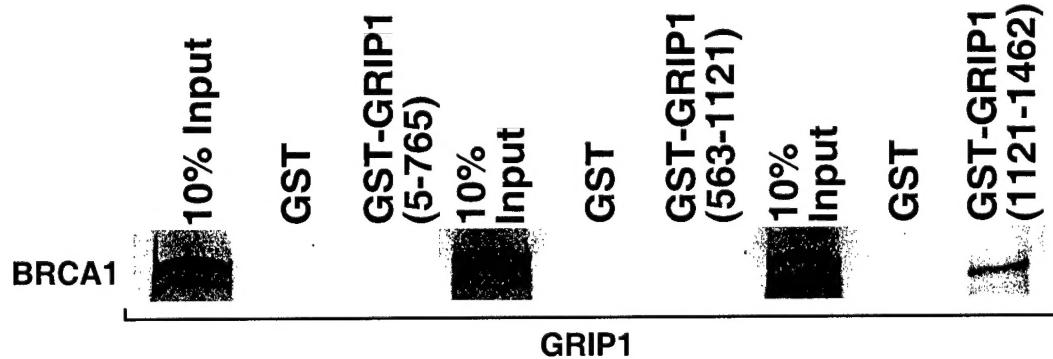


FIGURE 9

FIGURE 10

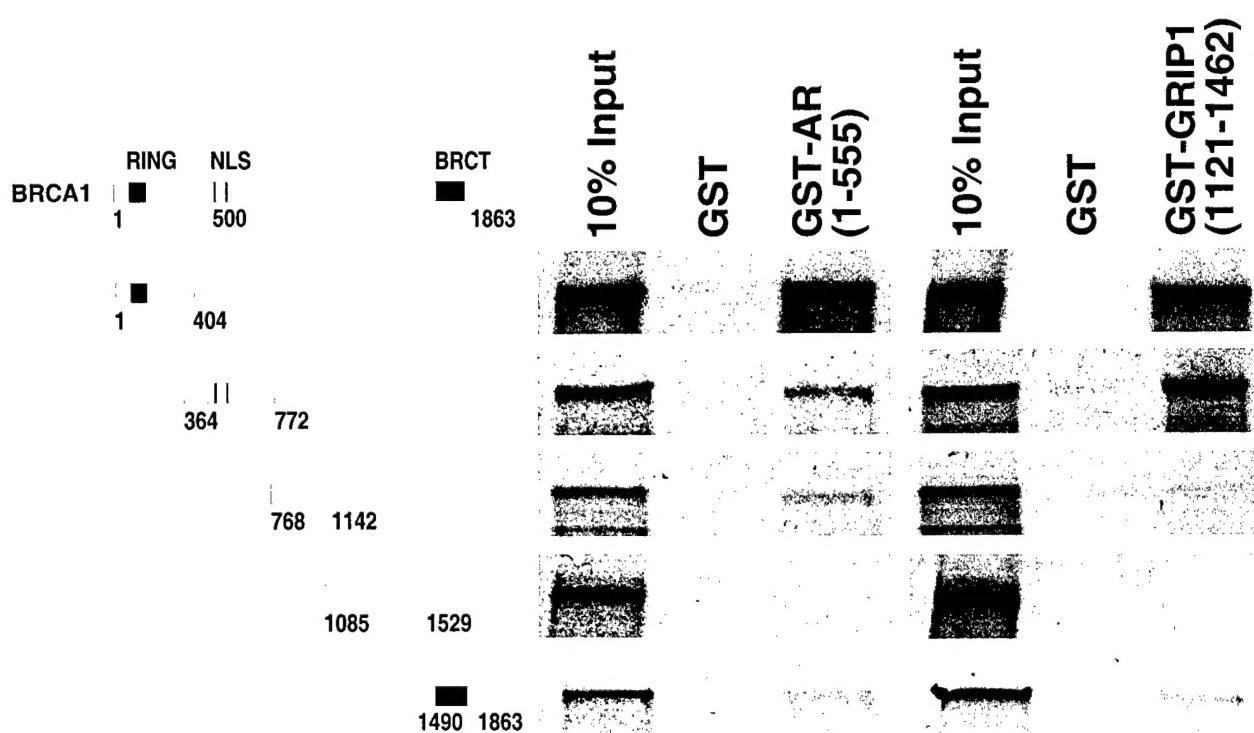
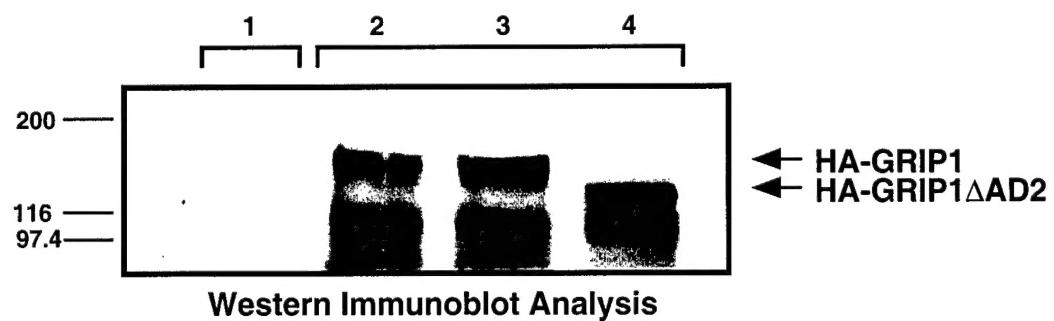
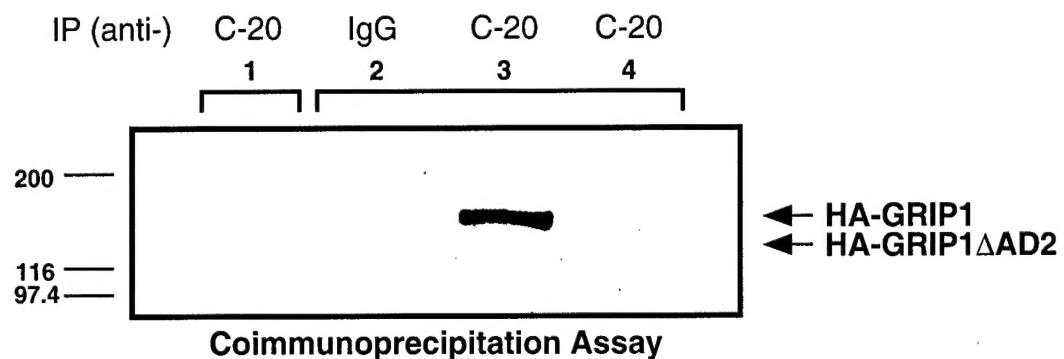


FIGURE 11



- 1 - Untransfected
- 2 - BRCA1/HA-GRIP1
- 3 - BRCA1/HA-GRIP1
- 4 - BRCA1/HA-GRIP1 Δ AD2

FIGURE 12

